Neuron, Vol. 45, 513–523, February 17, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2005.01.013

Differential Requirement for Plexin-A3 and -A4 in Mediating Responses of Sensory and Sympathetic Neurons to Distinct Class 3 Semaphorins

Avraham Yaron,[1,2] Pei-Hsin Huang,[3] Hwai-Jong Cheng,[1,3,*] and Marc Tessier-Lavigne[1,2,*]
[1]Department of Biological Sciences
Howard Hughes Medical Institute
Stanford University
Stanford, California 94305
[2]Genentech, Inc.
1 DNA Way
South San Francisco, California 84080
[3]Center for Neuroscience
Section of Neurobiology, Physiology, and Behavior
Division of Biological Sciences and
Department of Pathology and Laboratory Medicine
School of Medicine
University of California, Davis
Davis, California 95616

Summary

The class 3 Semaphorins Sema3A and Sema3F are potent axonal repellents that cause repulsion by binding Neuropilin-1 and Neuropilin-2, respectively. Plexins are implicated as signaling coreceptors for the Neuropilins, but the identity of the Plexins that transduce Sema3A and Sema3F responses in vivo is uncertain. Here, we show that Plexin-A3 and -A4 are key determinants of these responses, through analysis of a *Plexin-A3/Plexin-A4* double mutant mouse. Sensory and sympathetic neurons from the double mutant are insensitive to Sema3A and Sema3F in vitro, and defects in axonal projections in vivo correspond to those seen in *Neuropilin-1* and *-2* mutants. Interestingly, we found a differential requirement for these two Plexins: signaling via Neuropilin-1 is mediated principally by Plexin-A4, whereas signaling via Neuropilin-2 is mediated principally by Plexin-A3. Thus, Plexin-A3 and -A4 contribute to the specificity of axonal responses to class 3 Semaphorins.

Introduction

Axons are guided to their targets during development by cues in the extracellular environment. Several families of guidance molecules, including Netrins, Slits, Ephrins, and Semaphorins, as well as molecules that are traditionally thought of as morphogens, contribute to precise axonal guidance in both vertebrates and invertebrates (Tessier-Lavigne and Goodman, 1996; Dickson, 2002; Schnorrer and Dickson, 2004). Of these, the Semaphorins are a large family, comprising 19 secreted and membrane bound molecules in vertebrates (Pasterkamp and Kolodkin, 2003). Within this family, the six secreted proteins in class 3 are the best studied, and of these Sema3A and Sema3F are particularly well characterized for their effects on developing axons. Although initially discovered as axon repellents, it is now clear that class 3 Semaphorins can trigger a variety of responses, including axon pruning, dendrite attraction, and dendrite branching (Bagri et al., 2003; Campbell et al., 2001; Fenstermaker et al., 2004; Polleux et al., 2000).

The receptors that mediate repulsive responses to class 3 Semaphorins have been the subject of intense studies. The receptors for Sema3A and Sema3F have been shown to be heteromeric complexes including binding and signaling moieties. The binding moieties are now well established to be members of the Neuropilin family, which comprises two members in vertebrates and is not found in invertebrates (Bagri and Tessier-Lavigne, 2002). The two Neuropilins each bind a distinct subset of class 3 Semaphorins: thus, Sema3A preferentially binds neuropilin-1, whereas Sema3F preferentially binds neuropilin-2 (Chen et al., 1997; Giger et al., 1998; Takahashi et al., 1998). Both in vitro and in vivo studies have demonstrated an absolute requirement for Neuropilins in mediating axonal responses to Sema3A and Sema3F (Chen et al., 2000; Giger et al., 2000; Kitsukawa et al., 1997). However, Neuropilins have a very short cytoplasmic domain, suggesting that they form a complex with an additional coreceptor(s) that functions as a signaling moiety (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Nakamura et al., 1998).

The identity of the signaling coreceptors for the Neuropilins has been less firmly established, but strong candidates are provided by members of the Plexin family, which is conserved from worms to vertebrates. Plexins are transmembrane proteins containing a Semaphorin (Sema) domain followed by a cysteine-rich motif in their extracellular region and a conserved Plexin-specific Sex-Plexin (SP) domain in their cytoplasmic region. The nine vertebrate Plexins are divided into four subfamilies (A–D) based on structural features (Tamagnone et al., 1999; Puschel, 2002). The identification of Plexins as candidate Semaphorin receptors was made in the immune system, where Plexin-C1 was purified as a binding protein for the viral Semaphorin A39R from vaccinia virus (Comeau et al., 1998). This study led several groups to investigate whether Plexins are signaling receptors for membrane bound Semaphorins. In *Drosophila*, evidence was thus obtained that D-Plexin A is a functional receptor for the transmembrane Semaphorin Sema-1a/b, based on the finding that *D-Plexin A* mutants and *Sema-1a/b* mutants exhibit similar axon guidance defects in vivo and that Sema-1a/b can bind to D-Plexin A in vitro (Winberg et al., 1998). Similarly, in *C. elegans*, SMP-1/Sema1A has recently been shown to bind PLX1/Plexin1, and *smp-1* and *-2* function in the same genetic pathway as *plx-1*, consistent with Plexin1 being a functional Semaphorin receptor (Fujii et al., 2002; Dalpe et al., 2004). In vertebrates, several membrane bound Semaphorins were similarly shown to bind directly to Plexins: Sema 4D to Plexin-B1, Sema7A to Plexin-C1, Sema6D to PlexinA1 (Tamagnone et al., 1999; Toyofuku et al., 2004; Gu et al., 2005). A secreted

*Correspondence: hjcheng@ucdavis.edu (H.-J.C.); marctl@gene.com (M.T.-L.)

Semaphorin, Sema3E, has recently been shown to bind directly to Plexin-D1, an interaction that appears to mediate the effects of this Semaphorin in patterning the vasculature (Gu et al., 2005). However, whether these binding relations all reflect roles for the Plexins as functional receptors in vivo remains uncertain. However, the evidence argues against Plexin-C1 mediating the actions of Sema7A on axons (Pasterkamp et al., 2003).

The class 3 Semaphorins Sema3A and Sema3F do not, however, appear to bind Plexins directly, and Plexins have been proposed instead to function as signaling coreceptors in complex with binding moieties provided by Neuropilins, a possibility supported by the fact that all nine vertebrate Plexins interact with Neuropilins in transfected cell lines (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999). Initial evidence in support of the coreceptor model came from the finding that putative dominant-negative constructs provided by truncated Plexin-A1 or -A2 proteins could block Sema3A responses when introduced into neurons (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999). In addition, in transfected COS cells Plexin-A1 or -A2 but not -A3 was shown to mediate a contraction response of the cells to Sema3A (or Sema3F) when coexpressed with Neuropilin-1 (or Neuropilin-2, respectively) (Takahashi and Strittmatter, 2001). However, the significance of these studies for the normal physiological responses of neurons to Sema3A and Sema3F has been uncertain.

To determine which Plexins, if any, mediate the normal responses of embryonic sensory and sympathetic neurons to Sema3A and Sema3F, we studied the expression of all nine Plexins and identified Plexin-A3 and -A4 as the most likely candidates, as they are expressed at high levels in these neurons (Cheng et al., 2001). Through generation and analysis of a *Plexin-A3* knockout mouse, we showed that responses to Sema3F are completely dependent and responses to Sema3A partially dependent on Plexin-A3, thus contradicting the COS cell studies and supporting a model in which Plexin-A3 is a signaling coreceptor with Neuropilin-2 and, to a lesser extent, Neuropilin-1 (Cheng et al., 2001). However, our analysis also indicated that there must be additional transducers that mediate Sema3A and Sema3F responses. First, sympathetic and sensory neurons only partially lose their responsiveness to Sema3A. Second, we detected only mild defects of the peripheral sensory projections and cranial projections in the *Plexin-A3* mutants (Cheng et al., 2001), compared to those observed in *Neuropilin-1* or *Sema3A* mutant mice, in which sensory axons defasciculate and spread widely, and some aberrantly cross the midline (Kitsukawa et al., 1997; Taniguchi et al., 1997). Third, only a subset of the abnormalities that were found in the *Neuropilin-2* or *Sema3F* mutant mice were found in the *Plexin-A3* mutants, even though *Plexin-A3*-deficient sympathetic axons completely lose their responsiveness to Sema3F (Cheng et al., 2001); specifically, defects in trochlear and oculomotor axons, as well as defects in the anterior commissure observed in *Neuropilin-2* or *Sema3F* mutants (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003) were not seen in *Plexin-A3* mutants. These findings prompted us to ask whether another Plexin or yet some other receptor could account for the remaining responses. Plexin-A4 is a candidate for this role, based on its expression pattern (Cheng et al., 2001; Suto et al., 2003). However, other transmembrane proteins have also been suggested to contribute to Semaphorin responses. In the immune system, Semaphorin-4A enhances the activation and differentiation of T cells through Tim2, a member of the T cell immunoglobulin domain and mucin domain protein family, whereas Semaphorin-4D (CD100) binds and inhibits the activity of CD72, a known negative regulator of B cells, thereby enhancing B cells' responses in vivo (Kumanogoh et al., 2000, 2002). In the nervous system, a β1 integrin(s), not Plexin-C1, mediates Sema7A stimulation of axon outgrowth (Pasterkamp et al., 2003). Yet, other transmembrane proteins, including L1 CAM, Off-track, and Met have been suggested to participate in Semaphorin responses (Castellani et al., 2000; Giordano et al., 2002; Winberg et al., 2001). The results highlight the need to identify the unknown transducer(s) to understand how class 3 Semaphorin signals are transduced in the nervous system in vivo.

Here, we have tested directly whether Plexin-A4 accounts for the residual class 3 Semaphorin responses seen in neurons from *Plexin-A3* knockout mice, through generation and analysis of a *Plexin-A4* knockout mouse and a *Plexin-A3, -A4* double knockout mouse. Our results support the idea that Plexin-A3 and -A4 together mediate the responses to class 3 Semaphorins in sensory and sympathetic neurons, with Plexin-A4 being principally responsible for mediating responses to Sema3A via Neuropilin-1 and Plexin-A3 being principally responsible for responses to Sema3F via Neuropilin-2.

Results

Generation of *Plexin-A4* Single Mutants and *Plexin-A3/Plexin-A4* Double Mutants

Analysis of *Plexin-A3* mutant mice demonstrated that Plexin-A3 transduces only part of the Sema3A and Sema3F responses in vivo and in vitro, suggesting that other Plexin family members might be involved in the responses. In situ hybridization experiments have shown that *Plexin-A4* is strongly coexpressed with *Plexin-A3* in the dorsal root ganglia (DRG), the trigeminal ganglia (TG), and the superior cervical ganglia (SCG, a sympathetic ganglion) at the developmental stages when these neurons are responding to class 3 Semaphorins (Cheng et al., 2001; Suto et al., 2003). To examine the role of Plexin-A4 in Semaphorin signaling, we therefore generated a *Plexin-A4* null mouse.

Multiple attempts to target the *Plexin-A4* genomic region encoding the N terminus of Plexin-A4 failed for unknown reasons (data not shown). We therefore decided to delete the Plexin-A4 genomic region containing exons encoding the transmembrane domain. Homologous recombination in embryonic stem cells was performed using a targeting vector designed to replace exons 18 and 19 of the *Plexin-A4* genomic sequence with a cassette for neomycin expression (Figure 1A). In addition, an endoplasmic reticulum (ER) retention signal was added at the end of the truncated Plexin-A4 protein in order to prevent its secretion, followed by a myc epitope that would facilitate the detection of any

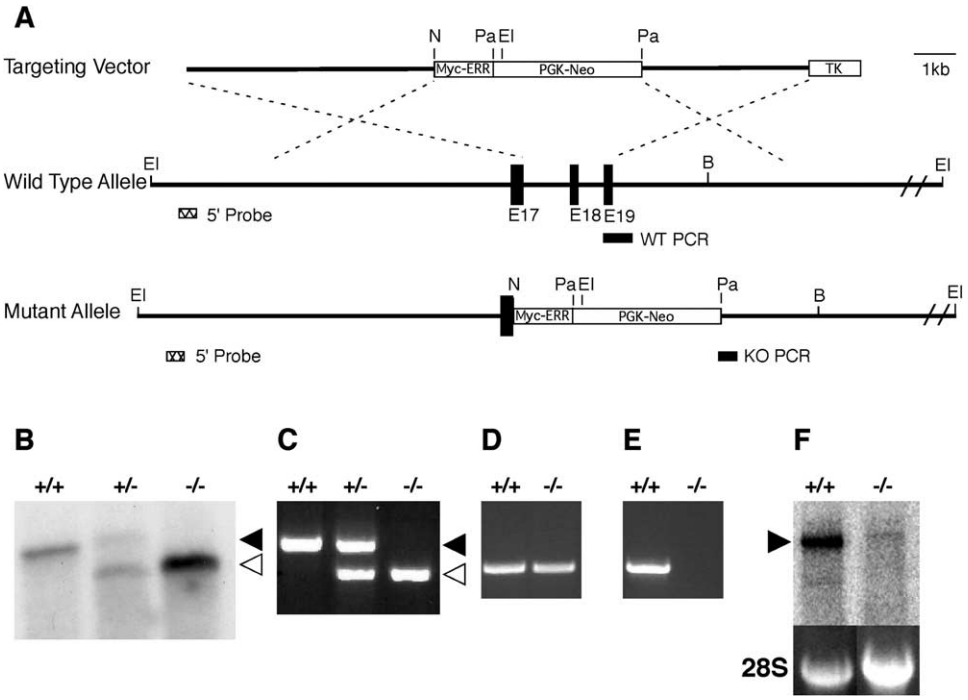

Figure 1. Generation of *Plexin-A4* Mutant Mice

(A) Schematic structures of targeting vector, wild-type *Plexin-A4* gene, and mutant allele. Homologous recombination should generate a mutant allele that lacks exon 19 (E19), which encodes the transmembrane domain, and exon 18 (E18). In addition, it will generate an in-frame insertion of a myc epitope followed by an ER retention signal (ERR) and a stop signal to the end of exon 17. The 5′ probe for Southern blot genotyping (cross-hatched boxes) and the PCR fragments specific for wild-type (WT PCR) and mutant (KO PCR) alleles are indicated. B, BamHI; EI, EcoRI; N, NotI; Pa, PacI.

(B) Southern blot analysis of tail genomic DNA of three offspring from an intercross of heterozygous mutants. Genomic DNA was digested with EcoRI and subjected to Southern blot analysis with the 5′ probe as indicated in (A). The sizes of the wild-type and mutant alleles are 17 and 10 kb, respectively. +/+, wild-type; +/–, *Plexin-A4* heterozygous mice; –/– *Plexin-A4* knockout mice.

(C) PCR genotyping of offspring from intercrosses of heterozygous mutants. The sizes of PCR products are 676 and 428 bp for the wild-type and mutant alleles, respectively.

(D and E) RT-PCR analysis of wild-type (+/+) and KO (–/–) RNA using primers 5′ to the cassette insertion (D) and 3′ to the cassette insertion (E).

(F) Northern blot analysis of wild-type (+/+) and KO (–/–) RNA using a 5′ probe. Neither the full-length wild-type *Plexin-A4* mRNA nor the truncated mRNA that could arise from the mutant allele was detected in the mutants. A very faint band with slightly higher molecular weight was noted in the mutant lane. The nature of this signal is unknown; however, if it represents some alternative *Plexin-A4* transcript, it would be at most 6% of wild-type (as assessed by phosphoimager analysis). Thus, the mutant is either a null or a near null. Solid arrowheads, wild-type bands; open arrowheads, mutant bands.

mutant protein that is made. Targeting events were detected by Southern blot (Figure 1B) and PCR analysis (Figure 1C).

Plexin-A4 homozygous mutants are viable and fertile. They are born in an approximate Mendelian ratio (16/70, 22%) from intercrosses of heterozygous mutants. We were able to detect mRNA for Plexin-A4 using a sensitive RT-PCR assay with primers designed to a region 5′ to the insertion of the Neo cassette but not with primers designed to a region 3′ to the cassette, indicating that only a shorter mutant message is generated (Figures 1D and 1E). The level of this message is, however, strongly reduced compared to wild-type, since it was not detected by Northern blot using a 5′ probe (Figure 1F), and since the truncated protein predicted to be translated from the mutant transcript is undetectable by Western blotting with anti-myc antibodies (see Figure S1 in the Supplemental Data available with this article online). Thus, we believe this allele is a null or near null allele, and we do not expect any significant dominant-negative effect of any truncated Plexin-A4 that is generated. This expectation was born out by careful examination of sensory axons from *Plexin-A4* heterozygous embryos both in vivo and through in vitro assays, as we did not detect any difference (compared to wild-type) in the trajectories of these axons in vivo or their responses to Semaphorins in vitro (data not shown).

To address whether Plexin-A3 and Plexin-A4 together are responsible for all Sema3A and Sema3F signaling, we also generated a *Plexin-A3*/*Plexin-A4* double knockout mouse (*Plexin-A3*/*A4* double) from crosses of single knockouts. To our surprise, we found that the double mutants are viable.

Sensory Axons from *Plexin-A3/A4* Double Mutant Mice Show Complete Loss of Responsiveness to Sema3A In Vitro

The axons of sensory neurons from the DRG of *Plexin-A3* mutant mice show only a slight reduction in response to Sema3A compared to wild-type neurons (Cheng et al., 2001). To address whether Plexin-A4

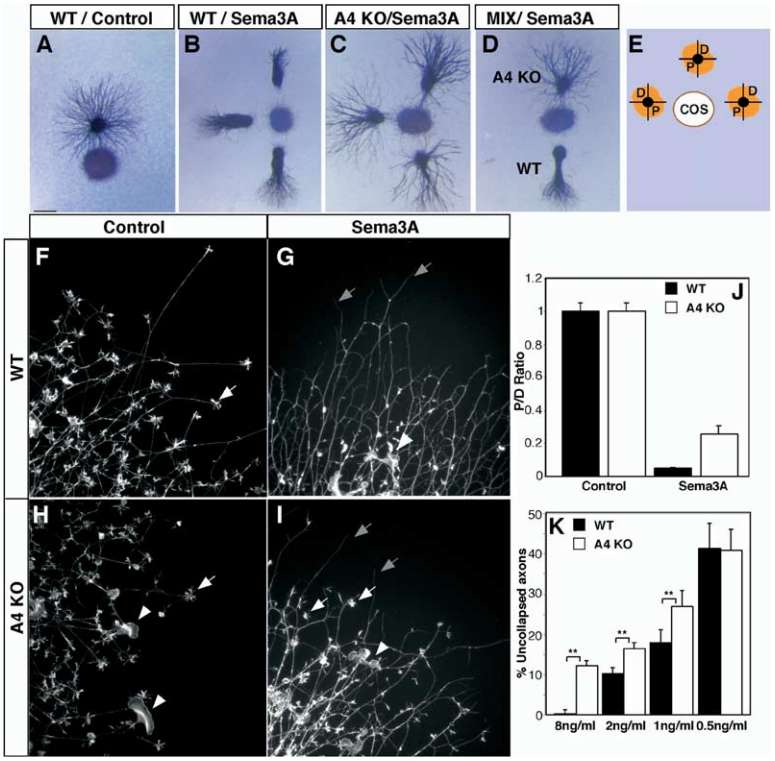

Figure 2. Plexin-A4 Is Required for Sema3A Signaling in DRG Axons

DRG axons from *Plexin-A4* mutant mice partially lose their response to Sema3A. E12.5 DRG were dissected and cocultured with cell aggregates in a collagen matrix. Mutant and wild-type explants were placed either separately (A–C) or in the same culture dish (D) to aid direct comparisons between explants. (J) shows quantification of representative repulsion experiments. The repulsive activity is measured by the axon outgrowth ratio P/D, where P is the extent of axon outgrowth on the side proximal to the cell aggregate, and D is the extent of axon outgrowth distal to the cell aggregate, as schematized in (E). A P/D ratio of 1 therefore indicates no repulsion. Standard error for each condition is indicated with a bar. For each experiment, six explants were used for each condition. Each set of experiments was repeated at least three times, and a representative is shown here. Scale bar is 150 μm. Explants were grown on a PDL/laminin surface and stimulated with 8 ng/ml of Sema3A for 45 min (F–I); white arrows indicate uncollapsed axons, gray arrows indicate collapsed axons, and arrowheads indicate migrating cells. (K) Dose response of wild-type and *Plexin-A4* mutant axons to Sema3A. Collapsed and uncollapsed axons were counted, and in each case the percentage of uncollapsed axons was calculated. For each experiment, about 100 axons were counted from four DRGs. Each experiment was repeated three times. $p < 0.0001$ for 8 ng/ml Sema3A, and $p < 0.04$ for 4 ng/ml and 2 ng/ml; Student's t test. DRG, dorsal root ganglion; WT, wild-type; A4 KO, *Plexin-A4* knockout mice.

might be mediating the remaining responses of *Plexin-A3*-deficient DRG axons, we examined the responses of DRG axons either from *Plexin-A4* single or *Plexin-A3/A4* double mutant mice in an explant collagen repulsion assay and a growth cone collapse assay.

Axons lacking *Plexin-A4* were still repelled by Sema3A (Figures 2C and 2D), but the amount of repulsion was clearly lower than what was observed with wild-type axons (Figures 2B–2D). First, approximately 10%–20% of the axons were not repelled at all and even grew directly into the aggregate of cells expressing Sema3A, suggesting that in some neurons Sema3A signaling may be transduced only by Plexin-A4. In addition, the remaining axons, while clearly repelled, fanned out broadly as they grew away from the Sema3A source, unlike wild-type axons, which were tightly fasciculated as they grew away. These results suggest that Plexin-A4 is an important but not exclusive mediator of the Sema3A response in these axons (Figures 2C and 2D). To quantify the response, we turned to growth cone collapse assays. At the concentration of Sema3A (8 ng/ml) that leads to 100% collapse of wild-type DRG growth cones, a small but significant portion of growth cones from *Plexin-A4*-deficient DRGs did not collapse (Figures 2G and 2I). When the concentration of Sema3A was reduced in the assays, a difference between wild-type and mutant growth cones was still observed, demonstrating that in the absence of Plexin-A4 some neurons lose their ability to respond to low concentrations of Sema3A.

We next examined the responses of the DRG axons from *Plexin-A3/A4* double mutants to Sema3A. Unlike the partial effects that were observed in *Plexin-A3* and *Plexin-A4* mutants, DRG axons that lacked both *Plexins* completely lost their responses to Sema3A in the explant collagen repulsion assay (Figures 3A–3C). When grown next to a COS cell aggregate secreting Sema3A, the double mutant axons exhibited radial outgrowth that was indistinguishable from that seen when axons were grown next to untransfected COS cell aggregates (compare Figure 2A to Figure 3C). To rule out the possibility that a small number of mutant axons might respond to Sema3A but that their responses were masked by the mass of unresponsive axons, we again turned to the growth cone collapse assay in which the response of each individual growth cone is not affected by neighboring axons and single growth cones are counted. We challenged the axons with a concentration of Sema3A that leads to 100% collapse of wild-type growth cones. In agreement with the repulsion assays, we found no difference in the number of collapsed growth cones between stimulated and nonstimulated axons from *Plexin-A3/A4* double mutant mice (Figures 3F and 3G). These results demonstrate that, in DRG axons, Plexin-A3 and Plexin-A4 are necessary signaling receptors for Sema3A. Other Plexins, although expressed at least at the mRNA level in some sensory neurons (Cheng et al., 2001; Murakami et al., 2001), do not appear sufficient on their own to mediate the responses.

Peripheral Sensory Axon Projections Are Severely Disrupted in *Plexin-A3/A4* Mutant Embryos In Vivo

We next examined the projections of sensory axons in the mutant embryos. Consistent with the in vitro data,

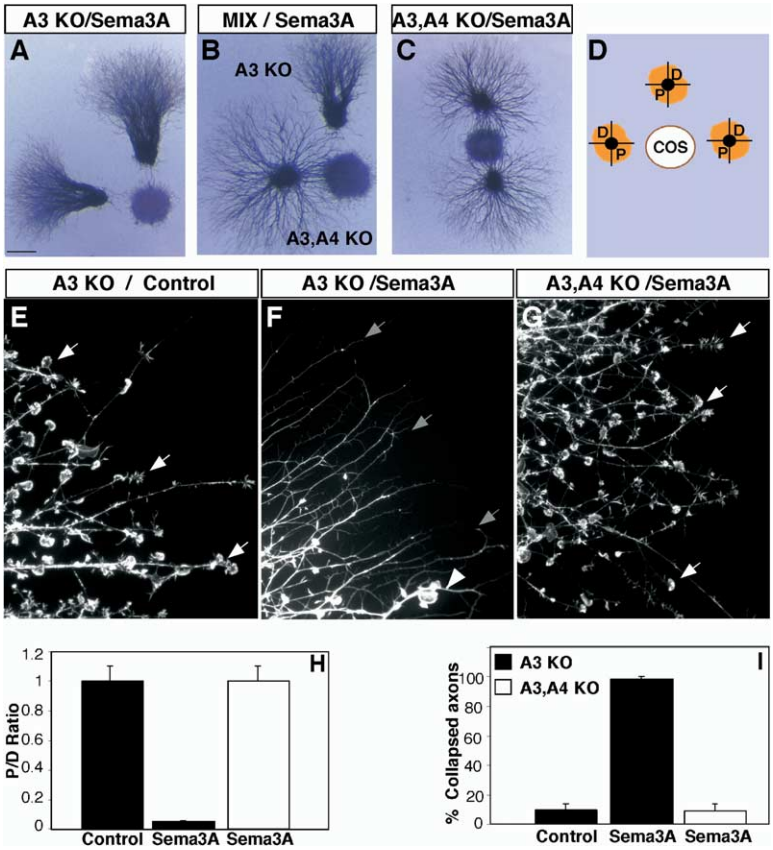

Figure 3. Plexin-A3 and Plexin-A4 Mediate Sema3A Signaling in DRG Axons

DRG axons from *Plexin-A3/A4* mutant mice completely lose their response to Sema3A. E12.5 DRGs were dissected and cocultured with cell aggregates in a collagen matrix. Mutant and control explants were placed either separately (A and C) or in the same culture dish (B) to aid direct comparisons between explants. As controls, DRGs from *Plexin-A3* mutant littermates' embryos were used. (H) shows quantification of representative repulsion experiments as described in Figure 2. Standard error for each condition is indicated with a bar; scale bar is 150 μm. Explants were grown on a PDL/laminin surface and stimulated with 8 ng/ml of Sema3A for 45 min (E–G and I). No difference was detected in the amount of collapsed growth cones between unstimulated controls and stimulated mutants. The quantification method is as described in Figure 2, but here the percentage of the collapsed axons is presented. DRG, dorsal root ganglion; A3 KO, *Plexin-A3* knockout; A3, A4 KO, double *Plexin-A3/Plexin-A4* knockout.

the peripheral sensory projections in the *Plexin-A3/A4* double mutants are severely disrupted. Exuberant arborizations were noted starting at E11.5 (Figures 4A and 4C). At E12.5, the abnormalities are even more dramatic (Figures 4D and 4F), with many axons actually crossing the dorsal midline to the other side of the body (Figures 4J–4L). The outgrowth and branching are so extensive at this stage that it is as if there are no more boundaries for the axons. The phenotypes observed in the double mutants are very similar to those observed in *Neuropilin-1* mutant embryos, providing evidence that the responses of sensory axons to Sema3A in vivo are mediated by both PlexinA3 and Plexin-A4 (Gu et al., 2003; Kitsukawa et al., 1997).

A mild but clear phenotype in spinal sensory neurons was observed in *Plexin-A4* mutants, indicating that Plexin-A4 by itself is partially required for the sensory projections in vivo. In the limb buds, the spinal sensory

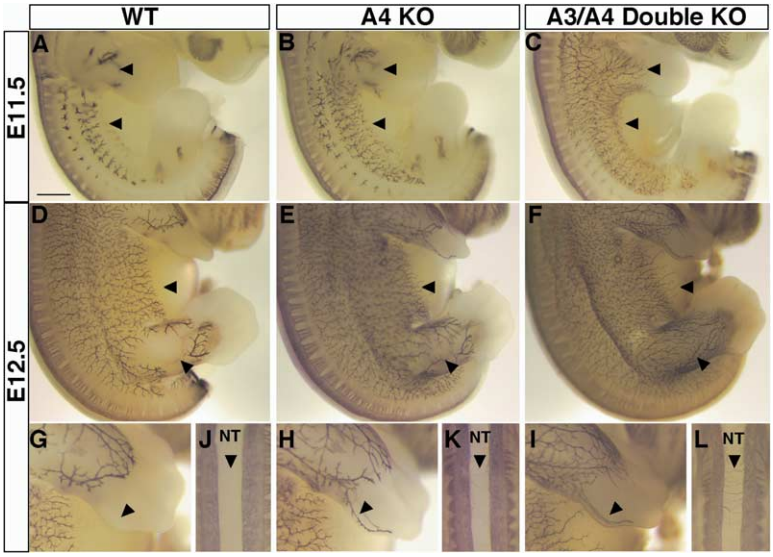

Figure 4. Severe Disruptions of Sensory Projections in *Plexin-A3/A4* Double Knockout Mice

Whole-mount anti-Neurofilament staining was performed on E11.5 and E12.5 embryos. Lateral view of embryos (A–F); dorsal is to the left. Peripheral sensory axons (arrowheads) in the *Plexin-A3/A4* mutant embryos exhibit severe arborization and defasciculation at E11.5 (A–C), a phenotype that is further enhanced at E12.5 (D–F). (G–I) Forelimbs at E12.5. Arrowhead indicates a misprojecting fiber bundle along the ventral part of the paw. (J–L) Neural tube (NT) at E12.5; dorsal view; anterior is up. Arrowhead indicates axons crossing the dorsal midline. WT, wild-type; A4 KO, *Plexin-A4* KO; A3/A4 double KO, *Plexin-A3/A4* double KO. Scale bar is 200 μm for (A)–(F) and 320 μm for (G)–(L).

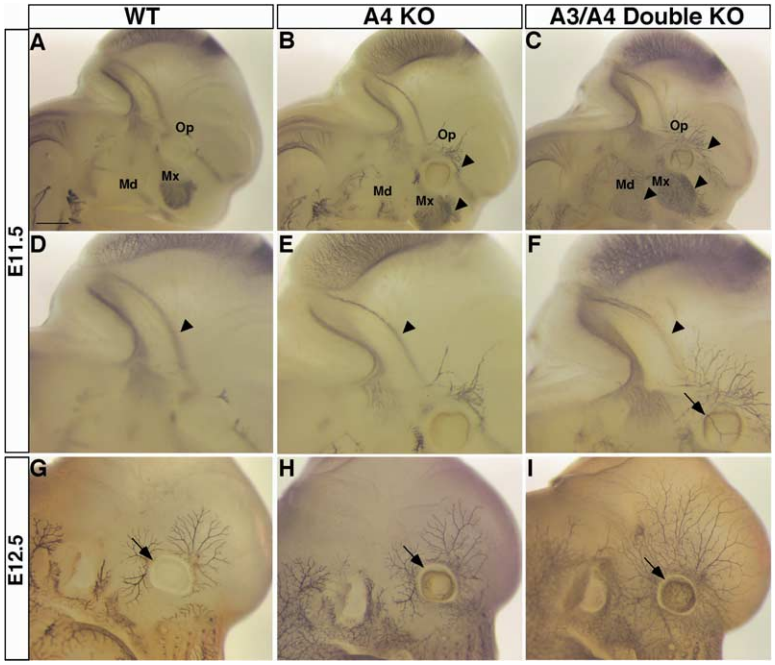

Figure 5. Severe Defects in Cranial Nerve Projections in *Plexin-A3/A4* Double Knockout Mice

Whole-mount anti-Neurofilament staining was performed on E11.5 and E12.5 embryos. Lateral view of embryos; dorsal is to the left. The three branches of the trigeminal nerve (V)—ophthalmic (Op), maxillary (Mx), and mandibular (Md)—exhibit disorganization in *Plexin-A4* KO and *Plexin-A3/A4* double KO mice (A–C). In the *Plexin-A4* KO, defects can be detected in the ophthalmic and the maxillary branches, as indicated by arrowheads. In the *Plexin-A3/A4* double KO, the disorganization is enhanced and can be detected in the mandibular branch as well. The trochlear nerve (IV), indicated by an arrowhead (D–F), is disrupted in the *Plexin-A3/A4* double KO, as a clear bundle can be detected in the wild-type and *Plexin-A4* KO, but only a few defasciculated fibers can be detected in the *Plexin-A3/A4* double KO. Fibers from the ophthalmic branch invade the eye region. Fibers can be detected already at E11.5 (arrow in [F]), and at E12.5 the entire eye is covered with crossing axons (G–I). Scale bar is 200 μm for (A)–(C) and (G)–(I) and 320 μm for (D)–(F).

axons in the *Plexin-A4* mutant mice failed to form thick nerve bundles at E11.5 (Figures 4A and 4B) and showed enhanced branching at E12.5 (Figures 4D and 4E). In addition, a misprojecting fiber bundle along the ventral part of the paw was observed in *Plexin-A4* mutant mice; this phenotype was not enhanced in the *Plexin-A3/A4* mutant mice and was not observed in *Neuropilin-1* mutant mice, suggesting that Plexin-A4 might be involved in guiding certain spinal sensory axons in a Neuropilin-1-independent fashion (Figures 4G–4I) (Gu et al., 2003; Kitsukawa et al., 1997).

It has been suggested previously that Sema3A might serve as a barrier in the ventral part of the spinal cord to restrict the central projections of NGF-sensitive DRG axons to the dorsal part of the spinal cord (Messersmith et al., 1995). We therefore examined the central projections of sensory axons into the gray matter of the spinal cord at E15.5 (data not shown) and E17.5 (Figure S1) in the *Plexin-A3/A4* double mutants by placing DiI in the DRG and anterogradely labeling the axons. No major defects were observed in these mutant animals, consistent with the lack of phenotype observed for these projections in *Sema3A* mutants as well (Taniguchi et al., 1997). It might be that additional repulsive cues exist in the ventral spinal cord or that local attractive cues in the dorsal spinal cord play a redundant and more important role in regulating this process.

Defects in the Cranial Nerve Projections in *Plexin-A4* Single and *Plexin-A3/A4* Double Mutants

Semaphorin signaling is also essential for the correct guidance of the cranial nerves. Our in vitro results suggested that defects should be observed in these projections in *Plexin-A4* mutant mice and, more severely, in *Plexin-A3/A4* double mutant mice. We analyzed mutant embryos at E11.5 and E12.5 using anti-Neurofilament (NFM) whole-mount immunostaining. In *Plexin-A4* mutants at E11.5, all three projections of the trigeminal ganglion (ophthalmic, maxillary, and mandibular) generally projected to their correct targets; however, clear disorganization of the ophthalmic and the maxillary projections was observed (Figures 5A and 5B). Most affected was the ophthalmic branch, which overshot beyond the front observed in wild-type embryos, with some fibers leaving the main bundle to grow in different directions. These defects were further enhanced in the *Plexin-A3/A4* double mutants. Much more severe disorganization and defasciculation of all three branches was detected, more fibers of the ophthalmic branch misprojected in different directions, and some of the fibers even started to invade the eye region (Figures 5A and 5C). In addition, the trochlear nerve was severely defasciculated, even though individual axons of this nerve bundle could still be seen leaving the midbrain-hindbrain junction (Figures 5D–5F). These latter results are very similar those observed in the *Neuropilin-2* and *Sema3F* mutants (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003).

At E12.5, axon fibers from the ophthalmic branch of the *Plexin-A3/A4* double mutant covered the entire face, including the eyes (Figures 5G–5I). The fibers were thinner and heavily branched when compared to those in the wild-type, demonstrating that these two Plexins mediate guidance, fasciculation, and branch suppression of these axons. Interestingly, in *Sema3A*, *Neuropilin-1*, or *Neuropilin-1*^{Sema} mutant mice, the eyes were not invaded by the aberrant axons as intensively as observed in the *Plexin-A3/A4* double mutants (Gu et al., 2003; Kitsukawa et al., 1997; Taniguchi et al., 1997). These results might indicate a Neuropilin-1-independent role for Plexins in guiding cranial nerves, as was suggested above for some spinal sensory nerves as well.

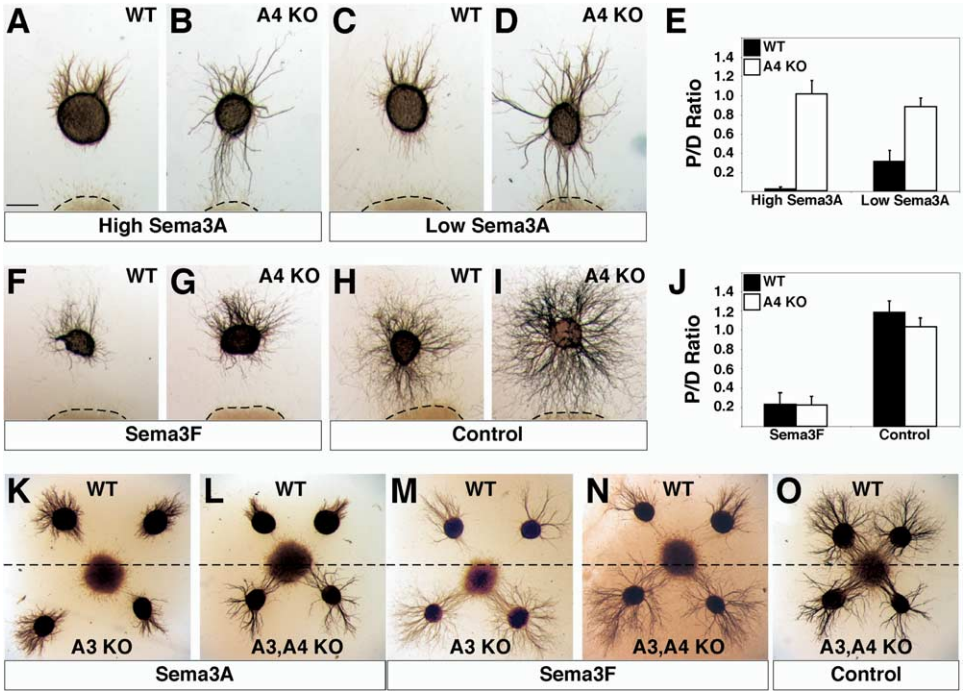

Figure 6. Plexin-A4 Is Required for Sema3A but Not Sema3F Signaling in Sympathetic Axons

E13.5 SCGs were dissected and cocultured with COS cell aggregates expressing Sema3A or Sema3F. (A–E) Repulsion of SCG axons by Sema3A is abolished in the *Plexin-A4* knockout mice. The repulsion was tested in two conditions with cell aggregates either from Sema3A-transfected COS cells (high Sem3A) or from Sema3A-transfected cells diluted with a 10-fold excess of untransfected COS cells (low Sema3A). SCG axons from the *Plexin-A4* mutant mice barely respond to Sema3A in both conditions ($p < 0.0001$ for high Sema3A, and $p < 0.01$ for low Sema3A; Student's t test); standard error for each condition is indicated with a bar. (F–J) SCG axons from *Plexin-A4* knockout mice were still repelled by Sema3F. (K–O) The SCG axons from *Plexin-A3/A4* double mutants (A3, A4 KO) lose their responses to Sema3A and Sema3F. In these assays, mutant and wild-type explants were placed in the same culture dish. For comparison, typical responses of the SCG axons from *Plexin-A3* knockout mice are included here (see also Cheng et al., 2001). WT, wild-type; A4 KO, *Plexin-A4* knockout mice; A3 KO, *Plexin-A3* knockout mice; A3, A4 KO, *Plexin-A3/A4* double knockout mice. Curved dotted lines in (A)–(D) and (F)–(I) indicate the position of cell aggregates. Straight dotted lines in (K)–(O) are used to separate the wild-type (top) and mutant (bottom) explants. Scale bar is 200 μm for (A)–(D) and (F)–(I) and is 430 μm for (K)–(O).

Responsiveness of Sympathetic Axons from *Plexin-A4* and *Plexin-A3/A4* Mutants to Class 3 Semaphorins In Vitro

Unlike sensory neurons, SCG neurons express both Neuropilin-1 and Neuropilin-2. Our previous study showed that Plexin-A3 was essential for signaling through Neuropilin-2 and that signaling through Neuropilin-1 was also partially affected, suggesting specificity in the association of Neuropilins with different Plexins. We examined the response of E13.5 SCG neurons from *Plexin-A4* mutants growing in the presence of NGF to Sema3A (Neuropilin-1 dependent) and Sema3F (Neuropilin-2 dependent) using the collagen repulsion assay. Interestingly, the result was a mirror image of that obtained with *Plexin-A3* mutants, i.e., whereas the response to Sema3A was partially reduced, the response to Sema3F was not affected (Figures 6A–6J). Next, we performed the experiment with neurons lacking both *Plexin-A3* and *Plexin-A4*. As found for DRG axons, the response to Sema3A was completely lost, and the axons demonstrated a radial outgrowth (Figures 6K–6O and Figure S2). Together with our previous demonstration that the response of SCG axons to Sema3F is completely lost in neurons from *Plexin-A3*-deficient animals, these results show that, as in sensory neurons, *Plexin-A3* and *Plexin-A4* mediate the response of sympathetic axons to class 3 Semaphorins.

Axon Guidance Defects in the Central Nervous System of *Plexin-A3/A4* Double Mutants

Several axon guidance defects in the central nervous system (CNS) were described in the *Neuropilin-2* and *Sema3F* mutant mice (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). Two clear phenotypes are the overshooting of the infrapyramidal bundle in the hippocampus and the disruption of the anterior commissure. In our previous work, we found that *Plexin-A3* is required for the Sema3F-dependent pruning of the infrapyramidal bundle (Bagri et al., 2003). However, we did not detect defects in the anterior commissure in the *Plexin-A3* mutant mouse (data not shown), unlike in *Neuropilin-2* mutant mice. We found clear disruption of the anterior commissure in the *Plexin-A4* mutants (Figure 7). The tract was reduced in size and defasciculated, as observed in *Neuropilin-2* and *Sema3F* mutant mice (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). These results support the idea that Plexin-A3

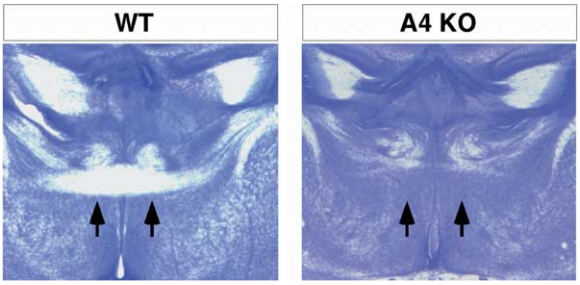

Figure 7. The Anterior Commissure Is Disrupted in the *Plexin-A4* KO

Coronal sections (100 μm) of E18.5 brains from wild-type and *Plexin-A4* KO mice were stained with cresyl violet. Arrows point to the anterior commissure. A thick bundle can be detected in the wild-type, but only a few fibers are present in the *Plexin-A4* KO (A4 KO).

and Plexin-A4 mediate the activity of class 3 Semaphorins in the CNS as well.

Discussion

The vertebrate class 3 Semaphorins are important regulators of axon guidance, cell migration, and vascular remodeling. Both in vitro and in vivo studies have shown that Neuropilin-1 and Neuropilin-2 are the binding moieties in class 3 Semaphorin receptors. However, the signaling receptors that partner with Neuropilin-1 and Neuropilin-2 in vivo to mediate the actions of class 3 Semaphorins are still poorly characterized. We have provided evidence that two members of the Plexin receptor family, Plexin-A3 and Plexin-A4, are the key necessary signaling receptors for class 3 Semaphorins in the guidance of a variety of developing axons in both the peripheral nervous system (PNS) and the CNS.

Plexin-A4 and -A3 Are Preferential Coreceptors for Neuropilin-1 and -2, Respectively

Initial in vitro studies identified the Plexins as coreceptors for Neuropilins. Several distinct Plexins have been shown to form complexes with Neuropilins in transfected cells, suggesting that all of them could potentially serve as coreceptors (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999). In COS cell-based assays, Plexin-A1, Plexin-A2, and Plexin-A4, but not Plexin-A3, were able to transduce a Sema3A and a Sema3F-induced signal when cotransfected with Neuropilin-1 or Neuropilin-2 (Suto et al., 2003; Takahashi and Strittmatter, 2001). However, our analysis of *Plexin-A3* knockout mice contradicted these in vitro studies and supported a role for Plexin-A3 both as an essential part of the receptor complex for Sema3F in sympathetic neurons and as a partial transducer of the Sema3A response in both sensory and sympathetic neurons (Cheng et al., 2001).

The fact that the Sema3A response showed only minor impairment in the *Plexin-A3* knockout directed our attention to the identity of other transmembrane proteins that could collaborate with Plexin-A3 in transducing the Sema3A signal. Our current analysis identifies Plexin-A4 as a key additional receptor, since sensory neurons from *Plexin-A4* knockout mice are strongly impaired in their Sema3A responses, and those from *Plexin-A3/A4* double knockout mice are totally insensitive to Sema3A, despite the fact that other members of the Plexin family are coexpressed in subsets of sensory neurons (Cheng et al., 2001; Takahashi et al., 1999). These results raise the possibility that Plexin-A3 and -A4 are the key Neuropilin coreceptors required for class 3 Semaphorin responses in sensory neurons, with Plexin-A4 being the principal coreceptor.

These results in sensory neurons are paralleled by those in SCG neurons. SCG axons express both Neuropilin-1 and Neuropilin-2 and thus respond to both Sema3A and Sema3F (Chen et al., 1998). Our previous study showed that the response to Sema3F is predominately dependent on Plexin-A3, whereas this study shows that the response to Sema3A is predominately dependent on Plexin-A4. These results confirm a degree of specific functional association of the members of the Plexin family with the two members of the Neuropilin family: at a functional level, Neuropilin-1 preferentially associates with Plexin-A4, and Neuropilin-2 preferentially associates with Plexin-A3 (Figure 8).

What accounts for this specificity? One possibility is that it could reflect a preferential physical association of Plexin-A4 and Plexin-A3 with Neuropilin-1 and Neuropilin-2, respectively. Previous studies did not report a preferential physical association of the different Plexins and the two Neuropilins as assessed by coimmunoprecipitation from transfected cells (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999); we have repeated such transfection studies and have observed a slight preference of Plexin-A3 for Neuropilin-2, though not of PlexinA4 for Neuropilin-1 (Figure S4). However, the presence or absence of a preferential interaction in transfected cells must be interpreted with caution, since the proteins are overexpressed; whether a preferential physical interaction exists under physiological conditions must therefore be addressed in primary neurons, especially since it could be promoted by host factors (such as proteins known to interact with Neuropilins, like L1 or other Ig superfamily members). The absence of antibodies that can be used to immunoprecipitate endogenous Plexins unfortunately makes this assessment impossible at this time. Alternatively, the preferential functional interaction that we observe need not reflect principally a direct preferential physical interaction of particular Plexins with particular Neuropilins but could reflect the operation of adaptor proteins that favor some interactions at the expense of others. In this context, it is of interest that members of the MICAL family, which participate in Semaphorin signaling, have been shown to associate differentially with members of the Plexin family, with hMICAL-1 and hMICAL-2 showing a preference for Plexin-A3 and Plexin-A4, respectively (Terman et al., 2002); whether these interactions contribute to the specificity that we observe remains to be determined. In addition, whichever model is correct (preferential physical interaction or action of cofactors), it is expected that the preferred partners of the Neuropilins could be different in different cells (see below).

Preferential Functional Interactions In Vivo

Our analysis of axon guidance defects in the peripheral sensory projections of *Plexin-A3* and *-A4* double mu-

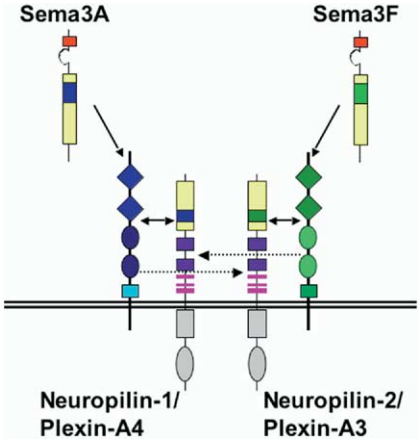

Figure 8. Preferential Association Model for Plexins and Neuropilins in Class 3 Semaphorin Signaling

Neuropilin-1 is the binding receptor for Sema3A, and Neuropilin-2 is the binding receptor for Sema3F. Plexins are coreceptors and signal transducers. In sensory and sympathetic neurons, the Sema3A/Neuropilin-1 signal is mediated principally by Plexin-A4, and the Sema3F/Neuropilin-2 signal by Plexin-A3. This preferential functional association is not, however, absolute, as the Sema3A/Neuropilin-1 signal can also be mediated partly by Plexin-A3, and the Sema3F/Neuropilin-1 signal partly by Plexin-A4. Horizontal arrows indicate the degree of functional interaction of Neuropilins and Plexins; it is not known whether they reflect preferential biochemical interactions of Neuropilin-1 with Plexin-A4 and Neuropilin-2 with Plexin-A3, or whether other host cofactors contribute or are entirely responsible.

tant embryos in vivo is in agreement with the model of preferential (though not exclusive) functional association of Plexin-A4 and Plexin-A3 with Neuropilin-1 and Neuropilin-2, respectively. First, the defects in sensory projections in the *Plexin-A3/A4* double mutant are very similar to the defects detected in the *Neuropilin-1* mutant (Gu et al., 2003; Kitsukawa et al., 1997), supporting the idea that, in vivo, Plexin-A3 and Plexin-A4 are required for mediating Sema3A signaling in these neurons. The similarity in the phenotypes also incidentally supports the idea that the major role of Neuropilin-1 in these neurons is as a receptor for class 3 Semaphorins rather than as an adhesion molecule. Importantly, axons from either *Plexin-A4* or *Plexin-A3* single mutants show partial loss of response to Sema3A, but the effect is more profound in the *Plexin-A4* mutants. Thus, *Plexin-A3* and *Plexin-A4* mutants may be thought of, respectively, as a weak functional hypomorph and a strong functional hypomorph of the *Neuropilin-1* mutant, at least for peripheral sensory projections. This is well illustrated in the case of the trigeminal ganglion: a mild defasciculation phenotype was observed in the ophthalmic branch in the *Plexin-A3* mutants, a more severe defect was detected in the *Plexin-A4* mutants, and the defects were further enhanced in the *Plexin-A3/A4* double mutants. The maxillary branch of the trigeminal ganglion was only mildly disorganized in *Plexin-A4* mutants but became more disorganized in the *Plexin-A3/A4* double mutants. Finally, the mandibular branch is disorganized only in the *Plexin-A3/A4* double mutants, not in either of the single mutants. The differential sensitivity of the three branches to the loss of *Plexin-A3* and *-A4* alleles is likely to be explained by the mandibular, maxillary, and ophthalmic branches being exposed to decreasing amounts of Sema3A protein, a possibility supported by decreasing levels of *Sema3A* mRNA in the environments of the three branches (Taniguchi et al., 1997). In any case, the in vivo phenotypes of the single and double mutants are in full agreement with the model that the Sema3A/Neuropilin-1 signal is transduced principally by Plexin-A4, and only to a lesser extent by Plexin-A3, in these neurons.

Another in vivo phenotype consistent with our model is the pruning of the infrapyramidal bundle in the hippocampus, which is mediated through the Sema3F-Neuropilin-2 pathway and which is severely impaired in the *Plexin-A3* mutant (Bagri et al., 2003; Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). (As evaluation of this defect requires the mutation to be on a CD-1 background, we have not yet been able to evaluate whether loss of *Plexin-A4* shows any impairment in this pruning.)

However, the in vivo evidence also supports the idea, mentioned above, that the specific functional interactions of Plexins and Neuropilins might be different in different cell types. First, pruning of the hippocampal-septal bundle, which is thought to be mediated through the Sema3A-Neuropilin-1 pathway, is highly dependent on Plexin-A3 (Bagri et al., 2003). Second, the development of the anterior commissure, which is disrupted in *Neuropilin-2* and *Sema3F* mutants, is defective in the *Plexin-A4* (as we show here) but not the *Plexin-A3* mutant (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). These observations are potentially consistent with either of the models discussed above. The cofactor model would simply require that the appropriate cofactors be expressed in hippocampal-septal and anterior commissural neurons. The preferential physical interaction model can also account for these observations if hippocampal-septal axons preferentially express Plexin-A3 rather than Plexin-A4, and if anterior commissure neurons have the opposite expression pattern. In this model, the preferential association rule (Plexin-A3 with Neuropilin-2, Plexin-A4 with Neuropilin-1) would reflect a biased competition when the Plexins are coexpressed, but when a single one of the Plexins is present it could pair with either one of the Neuropilins. If this is correct, then other Plexins might also function as Neuropilin coreceptors in other cells if they do not need to compete with Plexin-A3 and -A4 for interactions with the Neuropilins. One example of this may be provided by migrating cardiac neural crest cells, which are dependent on Sema3C and Sema3A and which require *Plexin-A2* for their migration; this could reflect selective expression of Plexin-A2 by these cells (Behar et al., 1996; Brown et al., 2001; Feiner et al., 2001).

Finally, two other in vivo phenotypes deserve mention. A striking observation in the peripheral sensory projections in the *Plexin-A3/A4* double mutant was the extensive branching of the aberrant axons. This branching phenotype may be secondary to the fact that the axons in the *Plexin-A3/A4* double mutants are also much longer than those in wild-type embryos. However, recent studies suggest that in cortical neurons Sema3A can directly inhibit branch formation without affecting axon length, which could also explain the enhanced branching that we observed (Dent et al., 2004). Another interesting phenotype in the double mutants is the

extensive growth and invasion of the aberrant axons into the eye region. This phenotype was not detected in *Neuropilin-1*, *Neuropilin-1*^{Sema}, or *Neuropilin-2* mutant mice, raising the possibility that this phenotype might reflect a Neuropilin-independent function of Plexin-A3 and -A4 in the eye (Chen et al., 2000; Giger et al., 2000; Gu et al., 2003; Kitsukawa et al., 1997). Interestingly, recent expression analysis of the class 3 Semaphorins in the cranial region in chicks demonstrated that Sema3A is expressed specifically in the eye, but such expression was not detected in the mouse (Chilton and Guthrie, 2003; Taniguchi et al., 1997).

In closing, our data support a model in which Neuropilin-1 associates preferentially but not exclusively with Plexin-A4 and Neuropilin-2 with Plexin-A3 to mediate axonal repulsion by Sema3A and Sema3F, respectively, in sensory and sympathetic neurons. Whether these preferential function associations reflect preferential physical association or the action of cofactors remains to be determined, as does whether other Plexins are used in other neuronal classes. Importantly, the *Plexin-A3/A4* double knockout is viable, providing a valuable tool to explore whether Semaphorin signaling contributes to axonal repulsion and inhibition not just in the embryo but also during regeneration of axons following injury.

Experimental Procedures

Generation of *Plexin-A4* Mutant Mice

The targeting vector was designed to replace exons 18 and 19 of *Plexin-A4* with a cassette containing PGK-neo. It contains a 6 kb homologous sequence upstream of exon 18, a 2 kb PGK-neo cassette, and a 4 kb homologous sequence downstream of exon 19 followed by the RSV-TK cassette.

To generate targeted ES cell lines, the vector was linearized and electroporated into E14 ES cells. Five correctly targeted ES cell lines were identified, and one gave good germline-transmitting male chimeras. Heterozygous females were generated and were used to establish lines that were backcrossed into either a C57BL/6 or a CD1 genetic background.

For Southern blot analysis, genomic DNA was digested by EcoRI and hybridized with a radiolabeled probe. A diagnostic 10 kb fragment indicated the mutant allele. For rapid genotyping, three primer PCR reactions were performed with a common antisense primer (5′-CTTCAGCACTGGCTGCTGTCATCT-3′), a sense primer for the wild-type allele (5′-CCATGCTCTCCTTCAGCCTGCTCT-3′), and a sense primer for the mutant allele (5′-GCTAAAGCGCATGCTCCA GACTGC-3′). The PCR products for wild-type and mutant were 676 bp and 428 bp, respectively.

Northern Blotting

Northern blot was done on total RNA from E12.5 embryos, using the NorthernMax kit. The first 1 kb of the coding region was used as a probe.

RT-PCR

RT-PCR was performed on RNA from E12.5 embryos with primers 5′ to the cassette insertion (5′-TGC ATC TTG AAC ATC CAG GGC ATC-3′, 5′-GCT CTC ATG GGC AGG GCA GTG CTG-3′) and 3′ to the cassette insertion (5′-AGC CCA GAG GTC CCA GTG AAG ATC-3′, 5′-CAG ATA GAT CTC AGA CAC CAT CTT-3′).

Explant Culture and Collapse Assay

Neuronal tissue explants from E13.5 SCG or from E12.5 DRG were cocultured with COS cell aggregates and stained as described (Chen et al., 2000). Collapse assays on axons from E12.5 DRG were performed as described (He and Tessier-Lavigne, 1997).

Immunohistochemistry and DiI Tracing

Whole-mount immunostaining using the 2H3 anti-NFM antibody on E11.5 and E12.5 embryos was performed as described (Chen et al., 2000). P1 brains and spinal cords were fixed, and DRG axon tracing experiments were performed as described (Taniguchi et al., 1997). Cresyl violet staining was performed on 100 μm vibratome sections of E18.5 brains.

Supplemental Data

The Supplemental Data include four figures and can be found with this article online at http://www.neuron.org/cgi/content/full/45/4/513/DC1/.

Acknowledgments

We thank B. Zhang, C. Ho, C. Sabatier, F. Charron, F. Wang, H. Long, X. Lu, J. Nguyen, M. Chen, and other members of the Tessier-Lavigne and Cheng laboratories for helpful discussions. We thank H. Rayburn and C. Jolicoeur for help in generating the knockout mice, and S. Faynboym for technical assistance. Special thanks to L. Ma for the help with the DiI injections and to R. Friedel for the help with the analysis of the anterior commissure. Part of this work was supported by a Whitehall Foundation grant (to H.-J.C.). A.Y. was supported by an HFSP fellowship. A.Y. was an Associate and M.T.-L. was an Investigator of the Howard Hughes Medical Institute.

Received: June 28, 2004
Revised: November 3, 2004
Accepted: January 9, 2005
Published: February 16, 2005

References

Bagri, A., and Tessier-Lavigne, M. (2002). Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. Adv. Exp. Med. Biol. *515*, 13–31.

Bagri, A., Cheng, H.J., Yaron, A., Pleasure, S.J., and Tessier-Lavigne, M. (2003). Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. Cell *113*, 285–299.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., and Fishman, M.C. (1996). Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature *383*, 525–528.

Brown, C.B., Feiner, L., Lu, M.M., Li, J., Ma, X., Webber, A.L., Jia, L., Raper, J.A., and Epstein, J.A. (2001). PlexinA2 and semaphorin signaling during cardiac neural crest development. Development *128*, 3071–3080.

Campbell, D.S., Regan, A.G., Lopez, J.S., Tannahill, D., Harris, W.A., and Holt, C.E. (2001). Semaphorin 3A elicits stage-dependent collapse, turning, and branching in Xenopus retinal growth cones. J. Neurosci. *21*, 8538–8547.

Castellani, V., Chedotal, A., Schachner, M., Faivre-Sarrailh, C., and Rougon, G. (2000). Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. Neuron *27*, 237–249.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547–559.

Chen, H., He, Z., Bagri, A., and Tessier-Lavigne, M. (1998). Semaphorin-neuropilin interactions underlying sympathetic axon responses to class III semaphorins. Neuron *21*, 1283–1290.

Chen, H., Bagri, A., Zupicich, J.A., Zou, Y., Stoeckli, E., Pleasure, S.J., Lowenstein, D.H., Skarnes, W.C., Chedotal, A., and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron *25*, 43–56.

Cheng, H.J., Bagri, A., Yaron, A., Stein, E., Pleasure, S.J., and Tes-

sier-Lavigne, M. (2001). Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron *32*, 249–263.

Chilton, J.K., and Guthrie, S. (2003). Cranial expression of class 3 secreted semaphorins and their neuropilin receptors. Dev. Dyn. *228*, 726–733.

Comeau, M.R., Johnson, R., DuBose, R.F., Petersen, M., Gearing, P., VandenBos, T., Park, L., Farrah, T., Buller, R.M., Cohen, J.I., et al. (1998). A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity *8*, 473–482.

Dalpe, G., Zhang, L.W., Zheng, H., and Culotti, J.G. (2004). Conversion of cell movement responses to Semaphorin-1 and Plexin-1 from attraction to repulsion by lowered levels of specific RAC GTPases in C. elegans. Development *131*, 2073–2088.

Dent, E.W., Barnes, A.M., Tang, F., and Kalil, K. (2004). Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. J. Neurosci. *24*, 3002–3012.

Dickson, B.J. (2002). Molecular mechanisms of axon guidance. Science *298*, 1959–1964.

Feiner, L., Webber, A.L., Brown, C.B., Lu, M.M., Jia, L., Feinstein, P., Mombaerts, P., Epstein, J.A., and Raper, J.A. (2001). Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. Development *128*, 3061–3070.

Fenstermaker, V., Chen, Y., Ghosh, A., and Yuste, R. (2004). Regulation of dendritic length and branching by semaphorin 3A. J. Neurobiol. *58*, 403–412.

Fujii, T., Nakao, F., Shibata, Y., Shioi, G., Kodama, E., Fujisawa, H., and Takagi, S. (2002). Caenorhabditis elegans PlexinA, PLX-1, interacts with transmembrane semaphorins and regulates epidermal morphogenesis. Development *129*, 2053–2063.

Giger, R.J., Urquhart, E.R., Gillespie, S.K., Levengood, D.V., Ginty, D.D., and Kolodkin, A.L. (1998). Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron *21*, 1079–1092.

Giger, R.J., Cloutier, J.F., Sahay, A., Prinjha, R.K., Levengood, D.V., Moore, S.E., Pickering, S., Simmons, D., Rastan, S., Walsh, F.S., et al. (2000). Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron *25*, 29–41.

Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., and Comoglio, P.M. (2002). The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat. Cell Biol. *4*, 720–724.

Gu, C., Rodriguez, E.R., Reimert, D.V., Shu, T., Fritzsch, B., Richards, L.J., Kolodkin, A.L., and Ginty, D.D. (2003). Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev. Cell *5*, 45–57.

Gu, C., Yoshida, Y., Livet, J., Reimert, D.V., Mann, F., Merte, J., Henderson, C.E., Jessell, T.M., Kolodkin, A.L., and Ginty, D.D. (2005). Semaphorin 3E and Plexin-D1 control vascular pattern independently of Neuropilins. Science *307*, 265–268.

He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell *90*, 739–751.

Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., and Fujisawa, H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron *19*, 995–1005.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., and Ginty, D.D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753–762.

Kumanogoh, A., Watanabe, C., Lee, I., Wang, X., Shi, W., Araki, H., Hirata, H., Iwahori, K., Uchida, J., Yasui, T., et al. (2000). Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. Immunity *13*, 621–631.

Kumanogoh, A., Marukawa, S., Suzuki, K., Takegahara, N., Watanabe, C., Ch'ng, E., Ishida, I., Fujimura, H., Sakoda, S., Yoshida, K., and Kikutani, H. (2002). Class IV semaphorin Sema4A enhances T-cell activation and interacts with Tim-2. Nature *419*, 629–633.

Messersmith, E.K., Leonardo, E.D., Shatz, C.J., Tessier-Lavigne, M., Goodman, C.S., and Kolodkin, A.L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron *14*, 949–959.

Murakami, Y., Suto, F., Shimizu, M., Shinoda, T., Kameyama, T., and Fujisawa, H. (2001). Differential expression of plexin-A subfamily members in the mouse nervous system. Dev. Dyn. *220*, 246–258.

Nakamura, F., Tanaka, M., Takahashi, T., Kalb, R.G., and Strittmatter, S.M. (1998). Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron *21*, 1093–1100.

Pasterkamp, R.J., and Kolodkin, A.L. (2003). Semaphorin junction: making tracks toward neural connectivity. Curr. Opin. Neurobiol. *13*, 79–89.

Pasterkamp, R.J., Peschon, J.J., Spriggs, M.K., and Kolodkin, A.L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. Nature *424*, 398–405.

Polleux, F., Morrow, T., and Ghosh, A. (2000). Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature *404*, 567–573.

Puschel, A.W. (2002). The function of neuropilin/plexin complexes. Adv. Exp. Med. Biol. *515*, 71–80.

Rohm, B., Ottemeyer, A., Lohrum, M., and Puschel, A.W. (2000). Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. Mech. Dev. *93*, 95–104.

Sahay, A., Molliver, M.E., Ginty, D.D., and Kolodkin, A.L. (2003). Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. J. Neurosci. *23*, 6671–6680.

Schnorrer, F., and Dickson, B.J. (2004). Axon guidance: morphogens show the way. Curr. Biol. *14*, R19–R21.

Suto, F., Murakami, Y., Nakamura, F., Goshima, Y., and Fujisawa, H. (2003). Identification and characterization of a novel mouse plexin, plexin-A4. Mech. Dev. *120*, 385–396.

Takahashi, T., and Strittmatter, S.M. (2001). Plexina1 autoinhibition by the plexin sema domain. Neuron *29*, 429–439.

Takahashi, T., Nakamura, F., Jin, Z., Kalb, R.G., and Strittmatter, S.M. (1998). Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat. Neurosci. *1*, 487–493.

Takahashi, T., Fournier, A., Nakamura, F., Wang, L.H., Murakami, Y., Kalb, R.G., Fujisawa, H., and Strittmatter, S.M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell *99*, 59–69.

Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G.I., Song, H., Chedotal, A., Winberg, M.L., Goodman, C.S., Poo, M., et al. (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell *99*, 71–80.

Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M., and Yagi, T. (1997). Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron *19*, 519–530.

Terman, J.R., Mao, T., Pasterkamp, R.J., Yu, H.H., and Kolodkin, A.L. (2002). MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. Cell *109*, 887–900.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123–1133.

Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Suto, F., Kamei, J., Aoki, K., Yabuki, M., Hori, M., Fujisawa, H., and Kikutani, H. (2004). Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. Genes Dev. *18*, 435–447.

Winberg, M.L., Noordermeer, J.N., Tamagnone, L., Comoglio, P.M., Spriggs, M.K., Tessier-Lavigne, M., and Goodman, C.S. (1998). Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell *95*, 903–916.

Winberg, M.L., Tamagnone, L., Bai, J., Comoglio, P.M., Montell, D., and Goodman, C.S. (2001). The transmembrane protein Off-track associates with Plexins and functions downstream of Semaphorin signaling during axon guidance. Neuron *32*, 53–62.